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Sexual dimorphisms in leukocyte trafficking in a mouse peritonitis model

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ABSTRACT

Sexual dimorphisms exist in the incidence and severity of many diseases, with females demonstrating relative protection from inflammatory conditions. The extent and mechanisms by which excessive leukocyte recruitment underlies these differences are not well established, and better understanding is essential for the development of targeted therapies. Here, we set out to compare the male and female inflammatory response in a murine zymosan-induced peritonitis model and to understand how leukocyte subsets are mobilized from storage pools in both sexes. This work shows that female C57BL/6 mice recruit fewer classical monocytes and neutrophils during zymosan-induced peritonitis. In addition, sex differences were evident in the circulation, as female mice showed reduced neutrophilia and monocytosis vs. male counterparts, despite having similar mobilization from BM stores. Importantly, we show that storage and trafficking of splenic leukocytes during acute inflammation are distinct between the sexes. Male mice have greater splenic stores of neutrophils and classical and nonclassical monocytes, despite similar spleen sizes, signifying another source of potential pathogenic leukocytes. This work demonstrates that males and females have distinct leukocyte-trafficking profiles in acute inflammation and suggests that the spleen, not the BM, plays a role in determining sex differences in the available pool of immune cells. Such dimorphisms demonstrate the importance of considering gender in assay development, drug design, and clinical trials. *J. Leukoc. Biol.* 98: 805–817; 2015.

Introduction

Inflammation is the natural response to infection, tissue damage, or irritation. In healthy individuals, this complex process serves to clear harmful stimuli and initiate tissue repair. However, under certain circumstances, this process can become dysregulated, causing an uncontrolled immune response that can lead to conditions, such as sepsis. These conditions are often debilitating and impose considerable social and economic burdens on the global population. The inflammatory response involves the endothelium and innate immune cells. The detrimental effects associated with inflammatory disease often stem from dysregulation of the mobilization and subsequent recruitment of inflammatory innate immune cells from sites of storage to the infection. Although many of the drivers and mechanisms of inflammation are understood, the impact of sexual dimorphism on this process remains unclear.

Sex differences in the inflammatory response have been described in diverse species. This is reflected in the severity of the response but also the resultant tissue damage [1]. In human disease, dimorphisms are regularly observed; men are more prone to inflammatory-type disorders, such as ischemia-reperfusion injuries and sepsis, and autoimmune diseases, such multiple sclerosis and rheumatoid arthritis, are more prevalent in women. A possible explanation for these differences is the effect of hormones. Estrogens, for example, are commonly proposed as protective in cardiovascular disease risk [2], although the exact mechanism is unclear.

There is also evidence that inherent differences in the immune system and innate cell phenotype are responsible for sexual dimorphisms in inflammatory responses. Female mice recruit fewer neutrophils upon detection of an inflammatory stimulus than age-matched males. This corresponds with females having more resident leukocytes in the peritoneal and pleural cavities [3]. Additionally, macrophages from females are more efficient at phagocytosis and reactive oxygen species production and are more abundant in pathogen-sensing TLRs than male counterparts [3]. Reductions in these characteristics were achieved by ovariectomy, signifying the influence of estrogens on immune cell phenotype. These observations

Abbreviations: ACK = ammonium-chloride-potassium, BM = bone marrow, C5aR = complement component 5aR, CX_3CR1 = CX3C chemokine receptor 1, eGFP = enhanced GFP, F = female, IL-1ra = IL-1R antagonist, M = male, MFI = median fluorescence intensity, PRR = pattern recognition receptor, qPCR = quantitative PCR, sICAM-1 = soluble ICAM-1, TIMP-1 = tissue inhibitor of metalloproteinase 1, Tx = transfer

The online version of this paper, found at www.jleukbio.org, *includes supplemental information.*

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suggest that females respond more effectively to, and clear infection faster than, males.

Although a mechanism for dampened female responses has been proposed, the reason for males having an exaggerated leukocyte recruitment response to an inflammatory stimulus is not clear. Such sex differences have clinical implications in immune system disorders, for which male and female patients currently receive equal treatments. As targeting estrogen signaling is an unlikely route for drug development as a result of the obvious hormone-imbalance effect, this study sought to explore further male-female differences in the inflammatory response. We investigated sex differences in the sources of recruited leukocytes by examining BM and spleen storage pools and probed further into the inflammatory response by also focusing on other leukocyte subsets that use the well-characterized murine zymosan-induced peritonitis model of resolving inflammation [4–6].

Under resting conditions, male mice had greater splenic reserves of neutrophils and classical and nonclassical monocytes than age-matched females. Previously, observed sex differences in neutrophil recruitment in peritonitis were confirmed in this study and extended to classical monocytes, which also accumulated, to a greater extent, in the peritoneal cavity of male mice. Despite mobilization from BM stores, evident in response to the inflammatory stimulus, no sex differences were observed. Males and females did, however, display different regulation of splenic neutrophil and monocyte trafficking in this model of inflammation. Uncovering such inherent differences in the male and female resident immune system and its response to infection should provoke more exploration into sex differences in human responses. In doing so, this will move us a step closer toward the development of personalized, sex-specific therapies, which are clearly required.

MATERIALS AND METHODS

Experimental animals

All animal procedures were carried out in agreement with the United Kingdom Home Office Animals Scientific Procedures Act 1986 under Project License 70/7884. C57BL/6 mice, 8–11 wk old, were used in this study (22–30 g males, 18–25 g females; Charles River Laboratories, Wilmington, MA, USA) and were housed in individually ventilated cages with a controlled 12 h light/dark cycle (lights on 7:00 AM to 7:00 PM) and constant temperature (21 ± 2°C). Mice from the same litter were distributed equally between experimental groups, and males and females were treated at the same time. In some experiments, mice heterozygous for targeted replacement of the CX_3CR1 gene with cDNA encoding eGFP ($CX_3CR1^{+/GFP}$) were used [7]. Male, 8 wk splenectomized, and sham-operated C57BL/6 mice were obtained from Charles River Laboratories, and animals were used 14 d postsurgery.

Induction of peritonitis and collection of leukocytes

Peritonitis was induced by intraperitoneal injection of 1 mg zymosan A (Lot #BCBF4506V; Sigma, St. Louis, MO, USA) in 0.5 ml sterile PBS after vortexing and 15 s sonication, as described previously [4]. After test periods, the animals were anesthetized by an intramuscular injection of 1 ml/kg ketamine (40 mg)/xylazine (2 mg) in saline and were euthanized by cervical dislocation, with care not to tear the carotid vessels. Leukocytes were obtained from blood by cardiac puncture by use of a 27G needle and syringe containing 50 μl 0.5 M EDTA; from the peritoneal cavity, by lavage with 6 ml wash buffer (PBS + 0.25% BSA + 2 mM EDTA); from BM, by flushing femurs through with ice-cold FACS buffer (PBS + 1% goat's serum); and from the spleen, by grating on a 40 μm cell strainer, followed by washing through with cold FACS buffer. Blood and spleen samples were subject to erythrocyte lysis with ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in dH_2O, pH 7.3), and all samples were washed twice with ice-cold FACS buffer by centrifugation (5 min, 300 g, 4°C).

Flow cytometry

Total leukocytes were counted by use of a hemocytometer with trypan blue (Sigma) for exclusion of dead cells and adjusted to a concentration of 1×10^6/ml in FACS buffer. Cells (1×10^5) were mixed with 0.5 μg anti-mouse CD16/CD32 Fc-block (BD PharMingen, San Diego, CA, USA) for 20 min on ice. Cells were immunostained for 30 min on ice in the dark with fluorescently conjugated antibodies to different leukocyte subsets, including CD3 (clone 17A2), B220 (clone RA3-6B2), and F4/80 (clone BM8; all BioLegend, San Diego, CA, USA); CD115 (clone AFS98); and Gr1 (clone RB6-8C5). Antibodies for the detection of murine cell surface receptors were included in certain experiments: TLR2 (clone T2.5), TLR6 (clone 4186-1), CCR1 (clone 643854), and CCR2 (475301; all R&D Systems, Minneapolis, MN, USA); dectin-1 (clone bg1fpj); CXCR4 (clone 2B11; eBioscience, San Diego, CA, USA); CXCR2 (clone TG11), CCR3 (clone J073E5), CCR5 (clone HM-CCR5), and C5aR (clone 20/70; all BioLegend); or respective isotype antibodies as controls. Cells were then washed twice by centrifugation (5 min, 300 g, 4°C) and resuspended in 200 μl FACS buffer for analysis by flow cytometry. Samples were acquired on the LSRFortessa (BD Biosciences, San Jose, CA, USA) by use of FACSDiva software (BD Biosciences) and compensation set by use of single-stained cells or anti-rat Ig, κ CompBeads (BD Biosciences). Data were analyzed by use of FlowJo (v7.6.4).

Chemokine and cytokine arrays

Chemokine and cytokine levels in peritoneal lavage from male and female mice in response to intraperitoneal zymosan were measured by use of Proteome Profiler Mouse Cytokine Kit (Panel A) and Mouse Chemokine Antibody Arrays, according to the manufacturer's instructions (R&D Systems). The resultant blots were scanned and quantified by densitometry by use of ImageJ.

Leukocyte transfer

Leukocytes were isolated from the BM of age-matched, untreated donor male and female C57BL/6 mice and were treated with ACK lysis buffer (5 min at room temperature). Male and female cells were labeled ex vivo with CellTracker Orange and CellTracker Violet dyes (2 μM; Life Technologies, Carlsbad, CA, USA), respectively. Labeled donor leukocytes (10×10^6; 5×10^6 male + 5×10^6 female) were transferred (intravenously) into recipient male or female mice. After 4 h, recipient mice were culled by cervical dislocation and spleen leukocytes harvested. Leukocytes were counted on a hemocytometer and labeled with antibodies recognizing CD115 and Gr1, and samples were analyzed by flow cytometry.

Cell sorting by flow cytometry and qPCR

Total leukocytes were prepared from spleens from CX_3CR1-eGFP heterozygote mice, as described. Leukocytes were stained with antibodies targeting GR-1, and leukocyte subsets were flow sorted by use of BD FACSAria (BD Biosciences). Leukocytes were identified by forward- and side-scatter characteristics and CD45-positive staining; dead cells were excluded by DAPI staining. Total mRNA from sorted neutrophils ($GR1^+$), classical monocytes ($CX_3CR1^+GR1^+$), and nonclassical monocytes ($CX_3CR1^+GR1^-$) from male and female spleen RNA were purified by use of the Qiagen RNeasy Micro Kit, as described by the manufacturer. cDNA was synthesized from 10 ng total RNA by use of the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), real-time qPCR was performed on

an ABI7900HT (Applied Biosystems; Life Technologies) real-time PCR machine, and reactions were prepared by use of iQ SYBR Green Supermix (Bio-Rad Laboratories). Gene expression was measured relative to housekeeping genes, and primers used in this study are described in Supplemental Table 1.

Statistical analysis

Data were expressed as means ± SEM. Statistical analysis was carried out by use of Prism 4.0 (GraphPad Software, La Jolla, CA, USA). For comparisons among multiple groups, a 1-way ANOVA was performed, followed by the Bonferroni posttest for multiple comparisons. For comparisons between 2 groups, the 2-tailed unpaired Student's *t* test was used.

RESULTS

Sex differences in leukocyte subset numbers under naïve conditions

A number of studies have reported differences in inflammatory responses between males and females [3, 8–10]. These studies have looked predominantly at cytokine levels and neutrophil responses to various stimuli. Here, we set out to expand these observations by comparing other leukocyte subsets between age-matched male and female mice. In an initial experiment, we established that there is no significant difference in total circulating, BM, or spleen leukocyte counts between male and female C57BL/6 mice (**Fig. 1A**). In line with previous reports [3], females exhibited significantly more peritoneal leukocytes than age-matched males (Fig. 1A; $4.4 \pm 0.20 \times 10^6$ vs. $3.3 \pm 0.20 \times 10^6$). This was found to be the result of significantly more peritoneal macrophages in females compared with males (Fig. 1B). To differentiate the populations of leukocyte subsets, we used C57BL/6 mice heterozygous for eGFP under the control of the CX_3CR1 promoter. Inflammatory responses in these reporter mice are identical to wild-type C57BL/6 mice [7]. Therefore, monocyte subsets could be detected by flow cytometry in conjunction with the anti-Ly6C/Ly6G antibody Gr1 in leukocyte preparations from blood, the peritoneal cavity, BM, and spleen. In alignment with previous studies [11], classical monocytes were identified as $CX_3CR1^+Gr1^+$, nonclassical monocytes as $CX_3CR1^+Gr1^-$, and neutrophils were $Gr1^{high}$. In addition, an indication of B and T cell numbers was achieved by use of B220 (CD45R) and CD3 as markers, respectively (gating strategies are defined in Supplemental Fig. 1A). With the use of this approach, we were able to compare numbers of leukocyte subsets from different compartments in male and female mice (Fig. 1). For the most part, neutrophils, classical and nonclassical monocytes, $B220^+$ cells, and $CD3^+$ cells showed no significant differences in the blood, peritoneal cavity, and BM (Fig. 1C–G). Peritoneal $B220^+$ cells from females were, however, more numerous than males, in line with other studies [3], and contributed to the greater numbers of total leukocytes found in the female peritoneum (Fig. 1F). Interestingly significantly fewer neutrophils and classical and nonclassical monocytes were present in the female spleen compared with the male (Fig. 1C and D; neutrophils: $18.7 \pm 2.00 \times 10^5$ vs. $44 \pm 7.94 \times 10^5$; classical monocytes: $7.7 \pm 0.82 \times 10^5$ vs. $16.1 \pm 2.49 \times 10^5$; nonclassical monocytes: $6.6 \pm 0.75 \times 10^5$ vs. $14.5 \pm 1.75 \times 10^5$). These differences were still evident even when spleen sizes and body weights were taken into account (**Table 1**). Together, these data suggest that there are sex differences with regard to basal leukocyte subset numbers in C57BL/6 mice.

Reduced monocyte and neutrophil responses in females during zymosan-induced peritonitis

Zymosan-induced peritonitis is an established model of inflammation [4]. Lower doses (1 mg) elicit a transient inflammation, whereas higher doses (10 mg) result in an aggressive response. To build on our previous observation of sex differences in leukocyte subsets under naïve conditions, we compared the response of leukocytes in a mild, resolving inflammatory scenario, induced with the use of a low 1 mg dose of zymosan. Analysis of leukocyte recruitment to the peritoneal cavity revealed that significantly fewer neutrophils were recruited to the site of inflammation in females compared with males, 3 h after zymosan injection (**Fig. 2A**; neutrophils: $8.0 \pm 0.91 \times 10^6$ vs. $11.4 \pm 1.12 \times 10^6$). Subsequent measurement of neutrophils over a full time course revealed that despite females demonstrating a dampened response, there was a clearance of neutrophils by 96 h in both sexes (Fig. 2A). Clearance of neutrophils at this time point is concurrent with previous reports [4] and indicative of resolution of inflammation. More classical monocytes were recruited after 3 h in male mice ($2.7 \pm 0.35 \times 10^5$ vs. $1.52 \pm 0.21 \times 10^5$), but this was not so of nonclassical monocytes that declined in number, roughly 2.5-fold in both sexes (Fig. 2B and C). Other leukocyte subsets, including $B220^+$ and $CD3^+$ cells, were not significantly different at this time point (Fig. 2D and E). Although there were a greater number of peritoneal macrophages in naïve females, the macrophage levels in both sexes were depleted substantially, 3 h after injection of zymosan (Fig. 2F).

Receptor expression and chemokine microenvironment

As we observed sex differences in neutrophil and classical monocyte recruitment to the peritoneal cavity in response to zymosan peritonitis, we next tested whether there were differences in the ability of peritoneal macrophages from males or females to sense and respond to the inflammatory stimuli, as has been proposed previously [3]. Detection of intraperitoneal zymosan occurs through the PRRs TLR2, TLR6, and dectin-1. The TLR2–TLR6 heterodimer is required for the inflammatory response and activation of NF-κB, and dectin-1 has roles in zymosan phagocytosis [12, 13]. No major differences were found in the expression of these receptors on the surface of peritoneal macrophages from male and female animals (Fig. 2G), suggesting that there are no sex differences in the ability of males and females to sense intraperitoneal zymosan.

Immune cells release a broad spectrum of chemokines and cytokines in response to inflammatory stimuli. We next examined whether the sex differences seen in neutrophil and classical monocyte recruitment could be explained by variances in the release of chemokines and cytokines in the tissue in response to zymosan. We used Proteome Profiler arrays to measure the levels

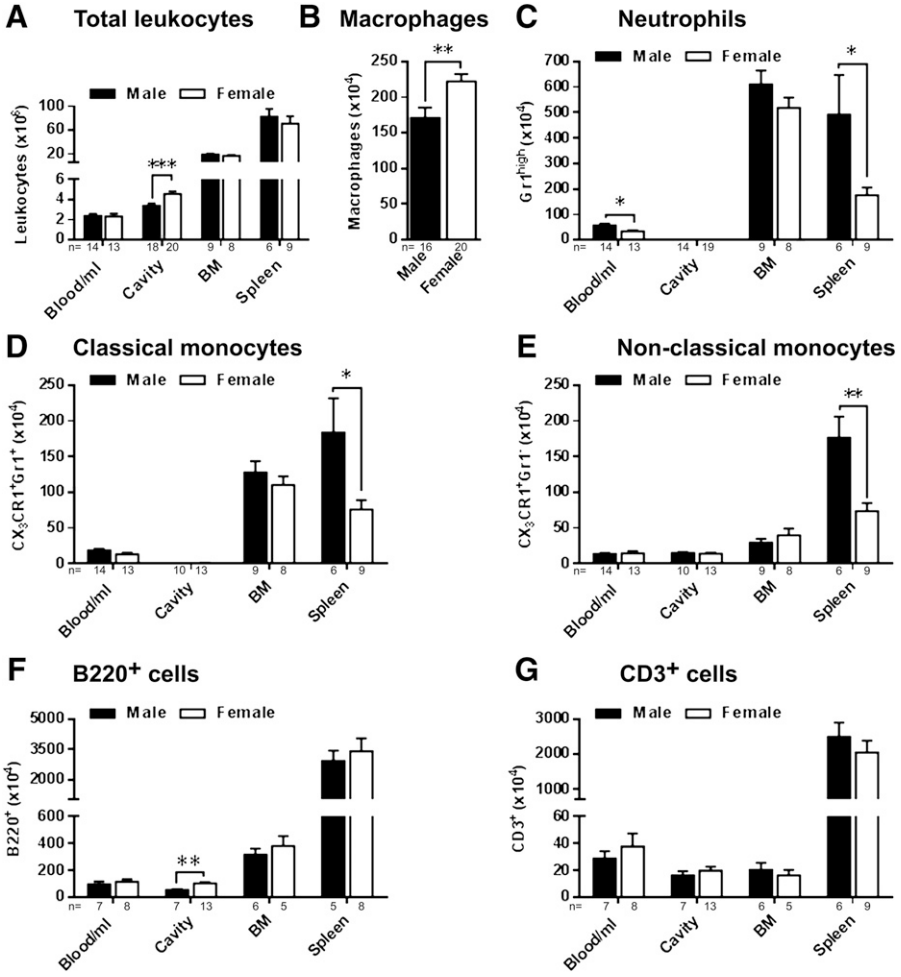


Figure 1. Sex differences in murine basal leukocyte subset numbers from different compartments. Numbers of cells corresponding to the indicated leukocyte subsets were counted from male (black bars) and female (white bars) C57BL/6 mice. Comparisons were made from leukocytes prepared from the blood, the peritoneal cavity, BM, and spleen. (A) Total leukocytes, (B) peritoneal macrophages ($F4/80^+$), (C) neutrophils ($Gr1^{high}$), (D) classical monocytes ($CX_3CR1^+Gr1^+$), (E) nonclassical monocytes ($CX_3CR1^+Gr1^-$), (F) $B220^+$ B cells, and (G) $CD3^+$ T cells. Numbers (n) for each measurement are shown below the bars in each graph. Data are shown as means ± SEM, and significant differences between sexes were determined by use of Student's t test and indicated by $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

of a broad spectrum of chemokines and cytokines in peritoneal lavage from naïve (Supplemental Fig. 1B) and inflamed mice. Peritoneal injection of zymosan resulted in a substantial increase in inflammatory mediators, such as C5a, G-CSF, sICAM-1, IL-1β, IL-1ra, IL-6, CXCL10, CXCL1, M-CSF, CCL2, CCL4, CXCL2, and TIMP-1. Other mediators, such as CCL6, chemerin, IL-16, CXCL1, CCL8, CCL12, and CCL9/CCL10, were also released in response to zymosan. There appeared to be no appreciable differences in the cytokine and chemokine profiles released in response to zymosan between males and females (Fig. 2H and I and Supplemental Fig. 1B).

Males recruit more neutrophils and classical monocytes to the blood during zymosan peritonitis despite no sex differences in leukocyte BM mobilization

As we could not observe any major differences in the peritoneal microenvironment between male and female mice, we next asked whether the differences observed in peritoneal leukocyte recruitment were related to the mobilization of leukocytes to the blood. Levels of circulating neutrophils and classical monocytes increased greatly, 3 h after zymosan administration, and returned to naïve levels after ~24 h (**Fig. 3A** and **B**). Interestingly and as seen with recruitment to the peritoneal cavity, circulating neutrophils and classical monocytes were significantly fewer in females compared with males, 3 h after the administration of zymosan (Fig. 3A and B). The profile of circulating nonclassical monocytes over time suggests that there was an increase in male-circulating numbers after 3 h; however, this was not observed in females (Fig. 3C).

The BM is a major leukocyte store in mice and humans and is reportedly used in peritonitis [14]. We speculated that the differences observed in neutrophil and classical monocyte numbers in the blood and peritoneal cavity in response to zymosan could be a result of dimorphisms in the way leukocytes are mobilized from the BM. CXCR4 plays as key role in this process [15], and we first established that there were no differences in the expression of this molecule between male and female BM leukocytes (data not shown). We then counted the number of leukocytes remaining in the BM (from the right femur) after intraperitoneal zymosan. After 3 h, the number of BM neutrophils and classical monocytes declined substantially, with levels restored to that of the naïve state, roughly 72 h postinjection (Fig. 3D and E). This indicated a rapid

TABLE 1. Murine body and organ characteristics

	Male	Female	*P*
Weight (g)	25 ± 2.58 (n = 99)	20.2 ± 2.16 (n = 94)	<0.001
Femur (mg)	81.6 ± 17 (n = 9)	76.4 ± 27.18 (n = 6)	NS
Femur: body weight (mg/g)	3.2 ± 0.74 (n = 9)	3.80 ± 1.42 (n = 6)	NS
Leukocytes: femur ($\times 10^5$/mg)	2.2 ± 0.78 (n = 6)	2.76 ± 0.60 (n = 5)	NS
Spleen (mg)	90.2 ± 19.24 (n = 15)	87.4 ± 25.12 (n = 17)	NS
Spleen: body weight (mg/g)	3.7 ± 0.62 (n = 17)	4.4 ± 1.01 (n = 17)	<0.05
Leukocytes: spleen ($\times 10^5$/mg)	10.4 ± 3.11 (n = 12)	9 ± 3.37 (n = 14)	NS

Measurements from naïve male and female mice. Data are displayed as means ± SEM of number (n) of experiments, as displayed in parentheses. Female data were compared with male by Student's t test and P value displayed. NS, not significant.

mobilization of both cell types in response to zymosan, which was to the same extent in males and females. Mobilization of nonclassical monocytes from the BM was equivalent in males and females; however, was not as substantial as the classical subset (Fig. 3F). Levels of nonclassical monocytes were replenished to naïve levels after 72 h. These data indicate that there are more circulating monocytes and neutrophils in males but no sex differences in mobilization of these cells from the BM during early zymosan peritonitis.

Sex differences in monocyte and neutrophil mobilization from the spleen

The spleen is another large store of leukocytes and has been shown as a major source of classical monocytes in certain inflammatory conditions [16–19]. We next set out determine whether there were any sex differences in the way monocytes and neutrophils are trafficked to and from this organ. We measured leukocyte numbers in the spleen from males and females in naïve and zymosan-treated mice (intraperitoneally; 3 h). Significantly fewer neutrophils and classical and nonclassical monocytes were present in the spleen of naïve female mice compared with males, as already observed (**Fig. 4A–C**). Zymosan had no effect on splenic neutrophil numbers; however, the number of female neutrophils roughly doubled in the spleen at this time point (Fig. 4A). Male classical monocyte numbers were greatly reduced, 3 h post-zymosan injection; however, female monocytes remained roughly consistent (Fig. 4B and C). Of importance, B and T cells represent the most abundant cell type in the spleen, and similar numbers are present in naïve and zymosan-treated (intraperitoneally; 3 h) male and female mice (Fig. 4D and E). This suggests that the observed sex differences relate primarily to neutrophils and monocytes and that males may use a splenic store of monocytes during inflammation, which may also account for the higher numbers of monocytes being recruited to the peritoneal cavity. To test this, we looked at leukocyte subsets in the blood of male splenectomized and sham-operated mice, 3 h after zymosan injection. We observed that there were significantly fewer neutrophils ($228 \pm 22.17 \times 10^4$ vs. $154.2 \pm 23.54 \times 10^4$) and classical monocytes ($105.7 \pm 13.6 \times 10^4$ vs. $67 \pm 4.5 \times 10^4$) in the blood in splenectomized animals compared with sham controls (Fig. 4F). No significant differences were observed in nonclassical monocytes and B and T cells. These experiments support the idea that in males, the spleen may be an additional pool of classical monocytes and neutrophils.

Sex differences in basal splenic leukocyte numbers are not related to the splenic chemokine microenvironment or chemokine receptor expression

To understand the underlying reason for more neutrophils and monocytes in the male compared with the female spleen, we first compared the basal splenic cytokine microenvironments. With the use of Proteome Profiler cytokine arrays, we showed that although a number of chemokines and inflammatory mediators were in abundance, e.g., CCL5, CXCL9, sICAM-1, IL-1ra, CXCL10, and IL-1α, there were no sex differences (**Fig. 5A**). In addition, it revealed the presence of those molecules with neutrophil or monocyte chemotactic capabilities, such as CCL2, CCL3, CCL5, C5a, CXCL1, and CXCL2, which were present in vastly ranging relative quantities but to similar extents in both sexes. The expression of the corresponding receptors to these inflammatory mediators on leukocyte subsets from the spleens of male and female mice was therefore assessed to offer explanation for the observed sex differences in splenic leukocyte composition.

CXCL1 and CXCL2 are potent neutrophil-attracting chemokines that exert their functions via CXCR2 [20]. Correspondingly, CXCR2 was highly expressed on the splenic neutrophil cell surface and expressed less on the surface of the other leukocyte subsets (Fig. 5B–D). No significant difference between the expression of CXCR2 on the surface of male and female splenic leukocytes was observed ($P > 0.05$). CXCL12, on the other hand, acts on CXCR4 on neutrophils but also on monocytes to retain these cells in the BM. CXCR4 was expressed at similar levels on the surface of neutrophils, monocyte subsets, and B cells from the spleen (Fig. 5B–D). Nonclassical monocytes from the female spleen, however, had significantly ($P < 0.05$) higher levels of CXCR4 than those from the male spleen (female MFI: 3003 ± 359.6; male MFI: 1821 ± 108.9; Fig. 5D).

Chemokines CCL2, CCL3, and CCL5 were all detectable, albeit at low levels, in the mouse spleen, as determined by cytokine array. CC chemokines are more promiscuous than those containing the CXC motif and exert their effects by binding to a selection of CCRs [21]. CCR1, CCR2, CCR3, and CCR5 represent key monocyte chemokine receptors that are

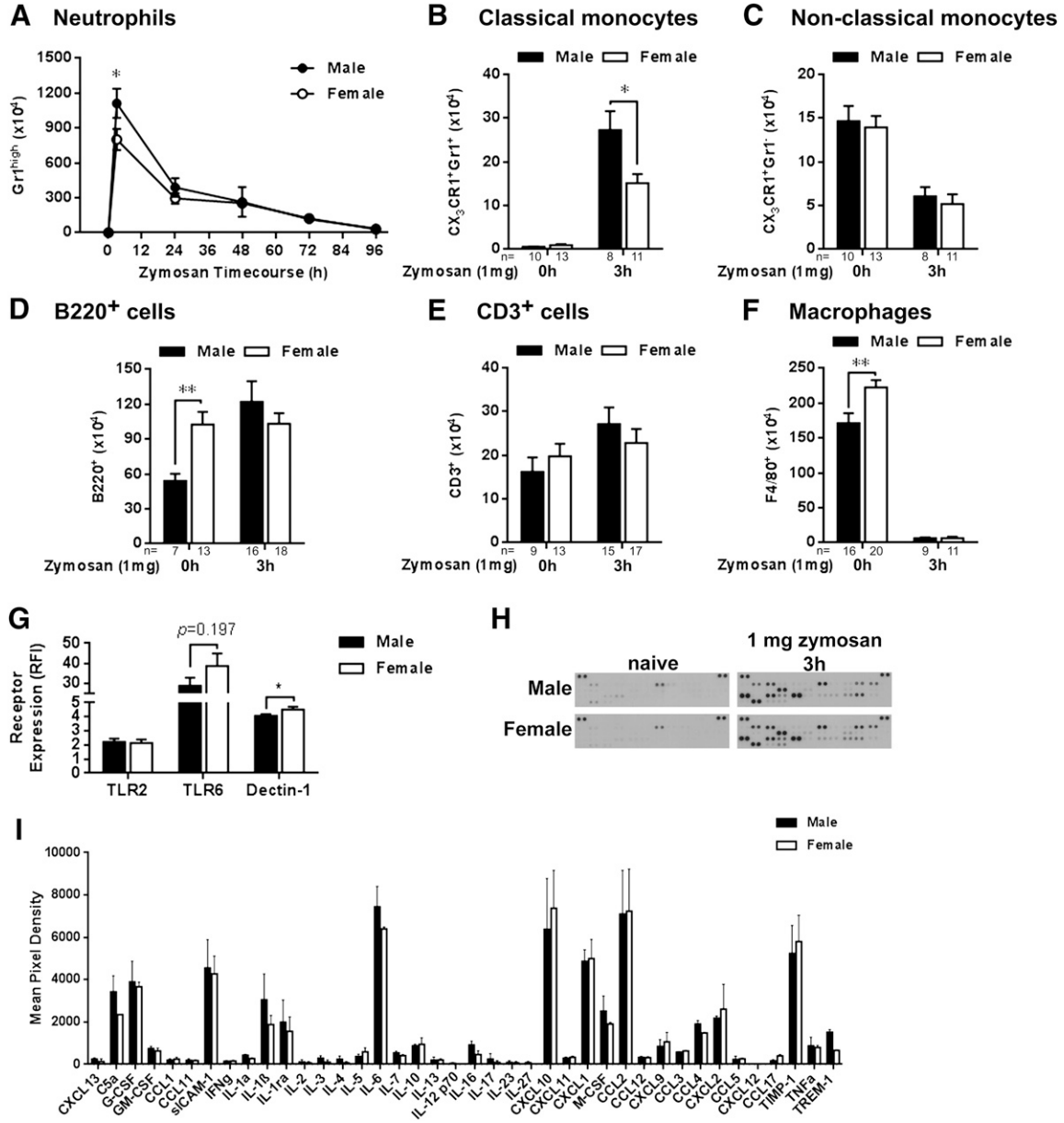


Figure 2. Sex differences in leukocyte recruitment to the peritoneal cavity in response to zymosan-induced peritonitis. Male (black circles and bars) and female (white circles and bars) C57BL/6 mice were treated with or without intraperitoneal zymosan. (A) Numbers of neutrophils ($Gr1^{high}$ cells) were measured over a period of 96 h. Data are shown as means ± SEM of at least 14 (0 h), 16 (3 h), 10 (24 h), and 3 (48, 72, and 96 h) mice. Sex differences in the accumulation of neutrophils in the peritoneal cavity at the indicated time points were determined by Student's *t* test. Significance in cell numbers in male compared with female mice is indicated by $*P < 0.05$. (B) Numbers of male and female classical monocytes ($CX_3CR1^+Gr1^+$), (C) nonclassical monocytes ($CX_3CR1^+Gr1^-$), (D) $B220^+$ cells, (E) $CD3^+$ cells, and (F) macrophages ($F4/80^+$ cells) were also determined after 3 h of peritonitis. Comparison of recruitment in male and female mice was evaluated by use of Student's *t* test, with significant differences at 3 h, indicated by $*P < 0.05$ or $**P < 0.01$. Data are displayed as the means ± SEM, and numbers (n) for each measurement are shown below the bars. (G) Expression of the zymosan receptors; TLR2, TLR6, and dectin-1 in peritoneal macrophages from male and female C57BL/6 mice. Expression of TLR2 ($n > 18$), TLR6 ($n > 14$), and dectin-1 ($n > 11$) was evaluated as relative fluorescence intensity (RFI) to an isotype antibody. Data are shown as means ± SEM and significant differences between sexes were determined by Student's *t* test by $*P < 0.05$. (H) Peritoneal cytokine profile in inflamed and naïve peritoneum. Representative Proteome Profiler array blots of peritoneal lavage from naïve and zymosan-injected (1 mg; 3 h) male and female C57BL/6 mice. (I) Semiquantification of Proteome Profiler blots by densitometry; data shown are the means of 3 experiments, and error bars reflect the SD. TREM-1, Triggering receptor expressed on myeloid cell 1.

associated with the above ligands [22]. CCR1 represents a receptor for an array of ligands, primarily chemotactic for monocytes, but in some cases, for neutrophils also [23]. Consequently, CCR1 was most highly expressed on the monocyte subsets and neutrophils. CCR2 is the major receptor responsible for chemotaxis of classical monocytes toward CCL2 [24, 25]. In the spleen, CCR2 was most highly expressed on classical monocytes, followed by the nonclassical subset,

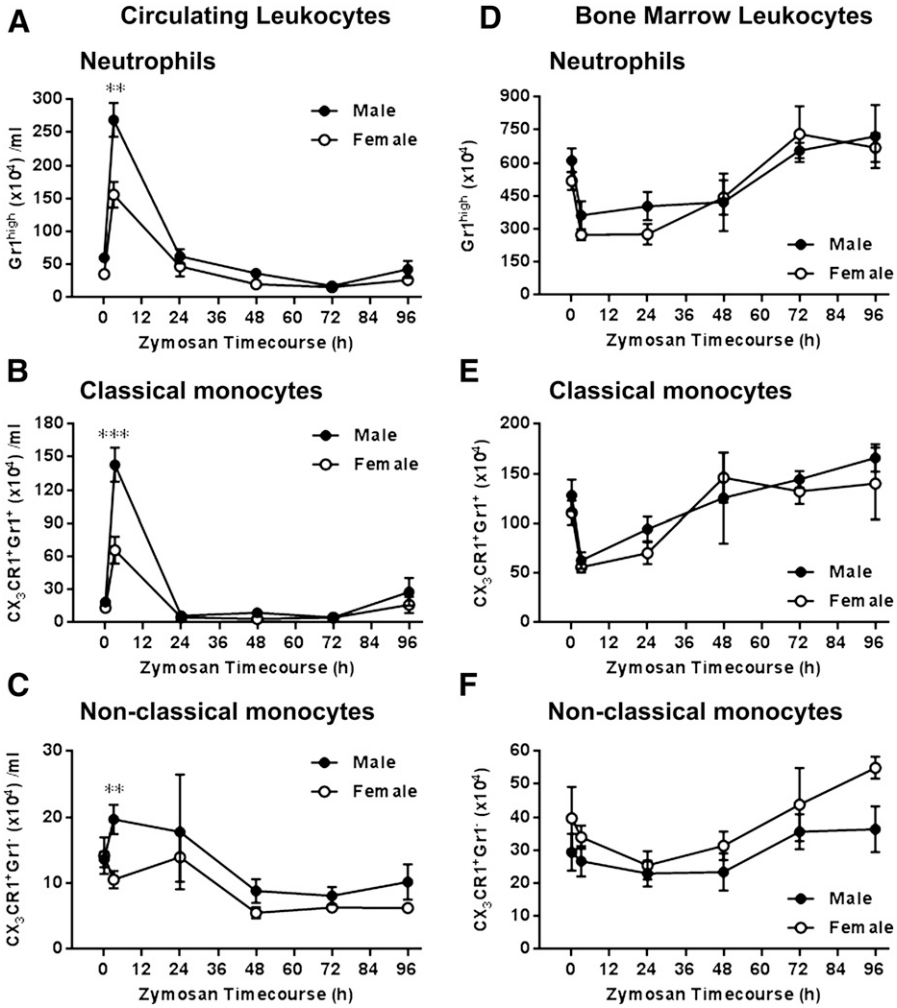


Figure 3. Sex differences in circulating leukocytes in response to zymosan peritonitis. Zymosan was administered to male (black circles) and female (white circles) mice to induce peritonitis. Numbers of (A) neutrophils ($Gr1^{high}$ cells), (B) classical monocytes ($CX_3CR1^+Gr1^+$), and (C) nonclassical monocytes ($CX_3CR1^+Gr1^-$) in the blood were counted at 0 (untreated), 3, 24, 48, 72, and 96 h. Data are presented as means ± SEM of at least 14 (0 h), 16 (3 h), 10 (24 h), and 3 (48–96 h) mice for each sex. Numbers of (D) neutrophils, (E) classical monocytes, and (F) nonclassical monocytes were counted from the right femur BM at the indicated time points. Data are presented as means ± SEM of 8 (0 h), 13 (3 h), 9 (24 h), and 3 (48–96 h) mice of each sex. Sex differences at each time point were determined by Student's *t* test and significance indicated by $^{**}P < 0.01$, and $^{***}P < 0.001$.

with very low expression on neutrophils (Fig. 5B–D). CCR3 is the receptor for chemokines, including CCL5, CCL7, CCL11, and CCL13, and CCR5, the receptor for chemokines, including CCL3, CCL4, and CCL5, showed low expression on all splenic leukocyte subsets studied (Fig. 5B–D). Notably, no sex differences in the expression of CCR1, CCR2, CCR3, or CCR5 were evident on splenic leukocyte subsets, with the exception of nonclassical monocyte CCR2, which was expressed more on cells from males (MFI: $20.6 \pm 1.01 \times 10^3$) vs. females (MFI: $13.0 \pm 1.69 \times 10^3$; $P < 0.01$; Fig. 5D). We analyzed further CCR2 mRNA levels in neutrophils and classical and nonclassical monocytes and found no obvious sex difference between male and females (Supplemental Fig. 2A).

As monocyte recruitment and deployment have been associated with angiotensin 2 [16], we next looked at the levels of expression of the angiotensin receptors AT1A and AT1B on naïve splenic neutrophils, classical monocytes, and nonclassical monocytes sorted by flow cytometry. No sex differences in gene expression were evident in any of the leukocyte subsets (Supplemental Fig. 2B and C). Additionally, we measured mRNA levels within these subsets of the major leukocyte adhesion receptors αM, αL, α4, β1, and β2 integrin and L-selectin, all of which showed no major differences between males and females in neutrophils or monocytes (Supplemental Fig. 3A–C). Finally, we compared the expression of C5aR between splenic leukocytes in males and females. C5a is a highly inflammatory peptide and represents an effective chemoattractant for neutrophils and monocytes via C5aR [26, 27], which was highly expressed on splenic neutrophils with moderate monocytic expression and with no apparent sex difference (Fig. 5B–D). Sex differences were evident on nonclassical monocytes, with female cells exhibiting significantly ($P < 0.01$) higher expression of C5aR vs. male cells (MFI: $4.6 \pm 0.35 \times 10^3$ vs. $2.8 \pm 0.34 \times 10^3$; Fig. 5C).

Inherent properties of male and female neutrophils and classical monocytes do not underlie sex differences in splenic leukocyte composition

To understand whether properties intrinsic to leukocytes from males and females or properties relating to the male and female body account for increased leukocyte numbers in the male spleen, leukocyte Tx experiments were performed.

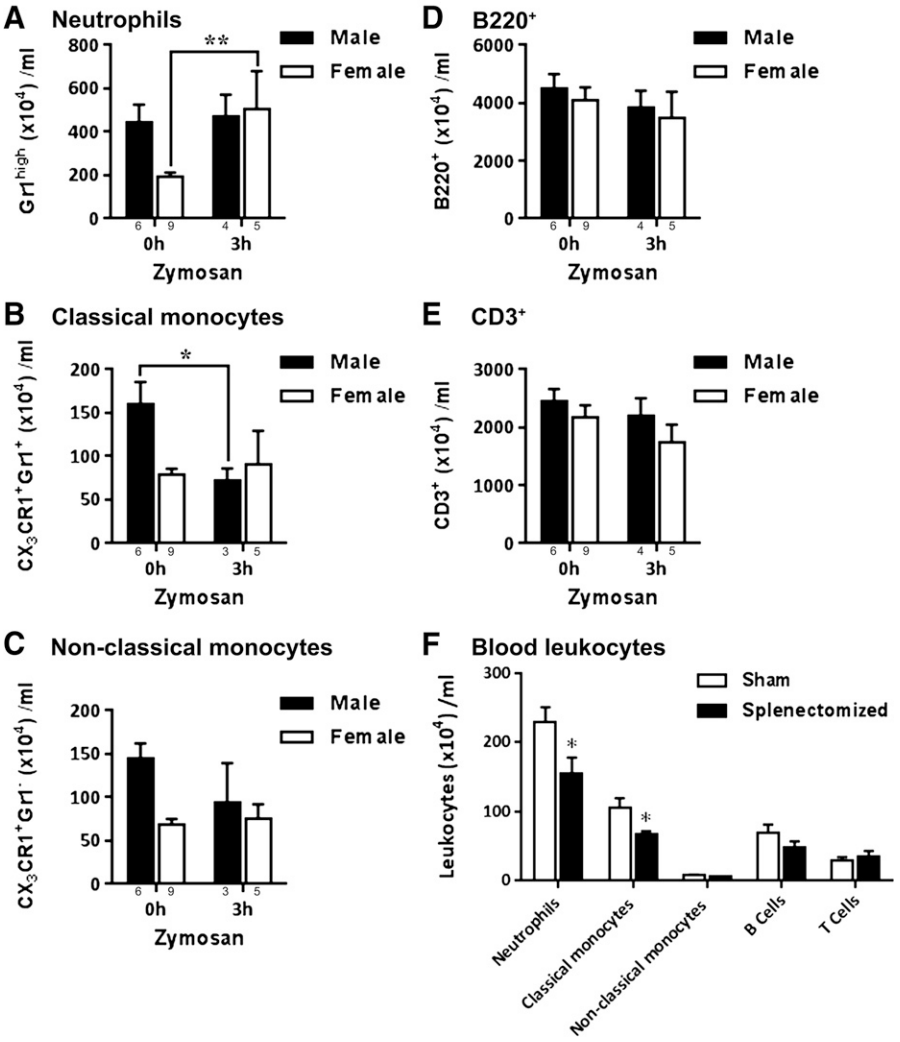


Figure 4. Splenic leukocyte trafficking during inflammation. Splenic (A) neutrophils ($Gr1^{high}$ cells), (B) classical monocytes ($CX_3CR1^+Gr1^+$), (C) nonclassical monocytes ($CX_3CR1^+Gr1^-$), (D) $B220^+$, and (E) $CD3^+$ cell numbers were determined in male (black bars) and female (white bars) mice, treated with or without zymosan intraperitoneally for 3 h. The influence of zymosan treatment on splenic leukocyte subset numbers was determined by Student's *t* test and significance indicated by $^*P < 0.05$ and $^{**}P < 0.01$. Data are expressed as means ± SEM, and numbers (*n*) are indicated below bars. Splenectomized male mice mobilize fewer neutrophils and classical monocytes to the blood (F). Sham-operated and splenectomized male mice ($n = 6$/treatment) were administered zymosan intraperitoneally, and after 3 h, leukocyte numbers in the blood were counted. Significant differences were calculated by use of a Student's *t* test between sham and operated animals ($^*P < 0.05$).

Equal numbers of male and female donor leukocytes were transferred to male and female recipient mice (intravenously), and their accumulation in the spleen was assessed. We proposed that equal accumulation of donor male and female neutrophils and monocytes in the recipient spleens would suggest that inherent properties of the spleen and body (e.g., chemokines and hormones) underlie the observed sexual dimorphisms in splenic leukocyte composition. Conversely, different accumulation of male compared with female donor leukocytes in the recipient spleen would be suggestive of inherent differences in the phenotype of the male and female neutrophil and monocyte. Comparable numbers of male (M) and female (F) total donor leukocytes accumulated in male recipient (M → M: 45.8 ± 9.7 × 10^4; F → M: 57.8 ± 27.5 × 10^4) and female recipient (M → F: 60.7 ± 11.9 × 10^4; F → F: 62.6 ± 19.6 × 10^4) mice (**Fig. 6A**), suggesting no adverse reactions in cross-sex donation. Irrespective of the sex of the recipient, a trend toward greater numbers of donor male neutrophils accumulated in the recipient spleen (M → M: 14.23 ± 5.2 × 10^4; M → F: 17.38 ± 4.3 × 10^4) compared with female donor cells (F → M: 8.6 ± 2.9 × 10^4; F → F: 11.16 ± 2.8 × 10^4; Fig. 6B). This was also the case with classical monocytes (M → M: 4.6 ± 0.9 × 10^4; M → F: 5.9 ± 1.4 × 10^4; F → M: 2.9 ± 0.6 × 10^4; F → F: 3.7 ± 1.0 × 10^4; Fig. 6C). However, these differences were not significant, and this coupled with the lack of any major differences in the expression of receptor molecules leads us to conclude that the difference in leukocyte numbers within the spleen is likely to be a result of inherent differences between the male and female body.

DISCUSSION

Substantial differences exist in the incidence and severity of certain inflammatory disorders, with males more prone to conditions with excessive or uncontrolled leukocyte recruitment and activation underlying their pathology (i.e., sepsis, ischemia/reperfusion injuries). The mechanisms behind these dimorphisms are unclear; however, previous studies report and link fewer resident macrophages expressing fewer PRRs to greater

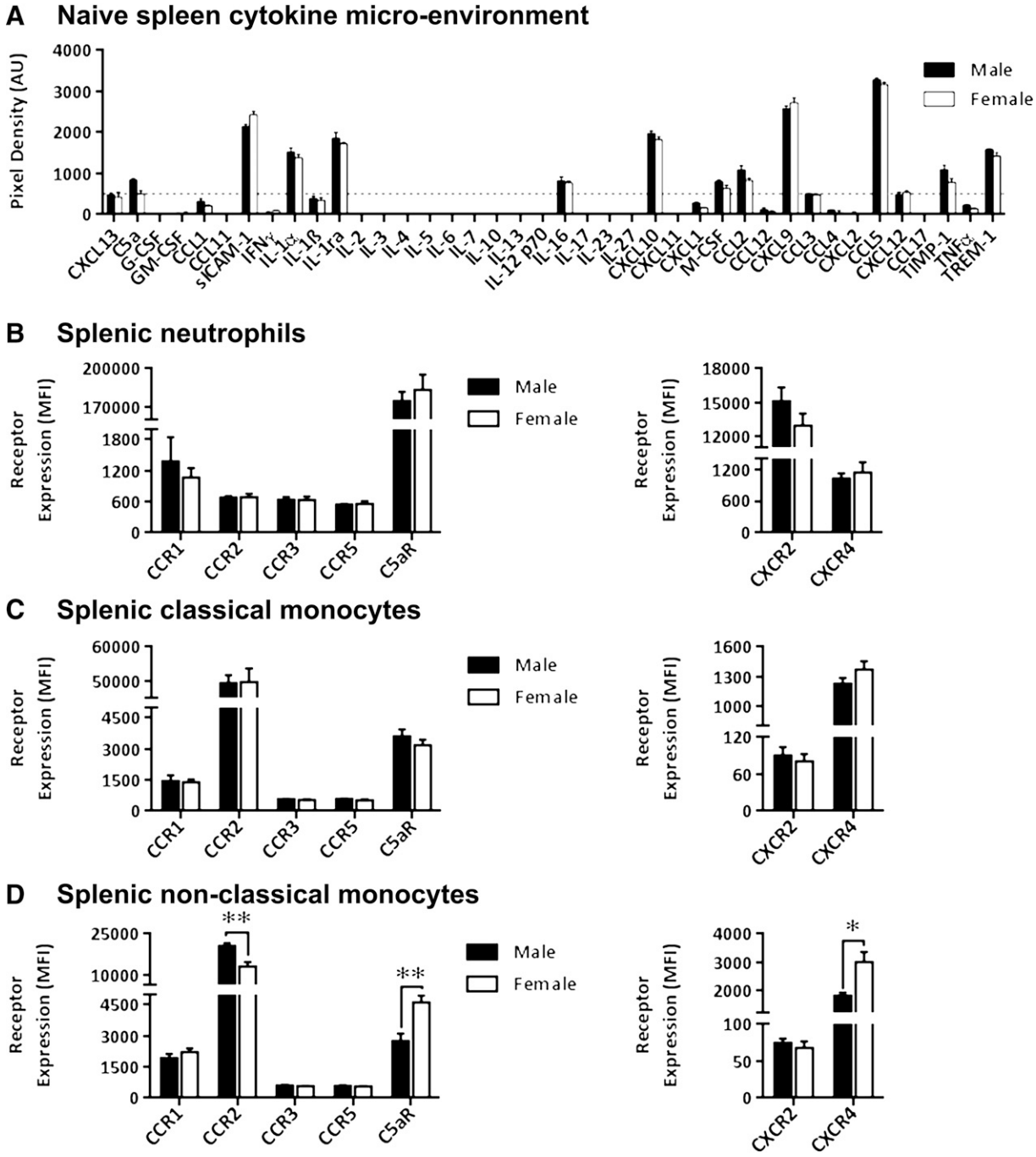


Figure 5. Basal splenic chemokine microenvironment and leukocyte receptor expression. (A) The chemokine profile of splenic lysates from untreated male (black bars) and female (white bars) C57BL/6 mice was compared by use of a Proteome Profiler chemokine array. Blots were quantified by densitometry, and the data shown are the mean of experiments, with error bars reflecting the highest and lowest values obtained. (B–D) Leukocytes were isolated from male and female spleens and cell surface expression of the CCRs CCR1, CCR2, CCR3, CCR5, C5aR, CXCR2, and CXCR4 were measured on (B) neutrophils, (C) classical monocytes, and (D) nonclassical monocytes. Receptor expression is displayed as MFI, as data are the means ± SEM ($n = 6$). Sex differences in receptor expression were determined by Student's t test and significance indicated by $^*P < 0.05$ and $^{**}P < 0.01$.

neutrophil recruitment in male mice in the zymosan peritonitis model [3]. In this study, we expand on this current knowledge of sexual dimorphisms in immune cell trafficking in acute inflammation. We show that like neutrophils, classical monocytes also accumulate to a greater extent in male C57BL/6 mice during zymosan peritonitis. Previous studies have shown that infiltration of classical monocytes is independent of neutrophil migration in peritonitis [28],

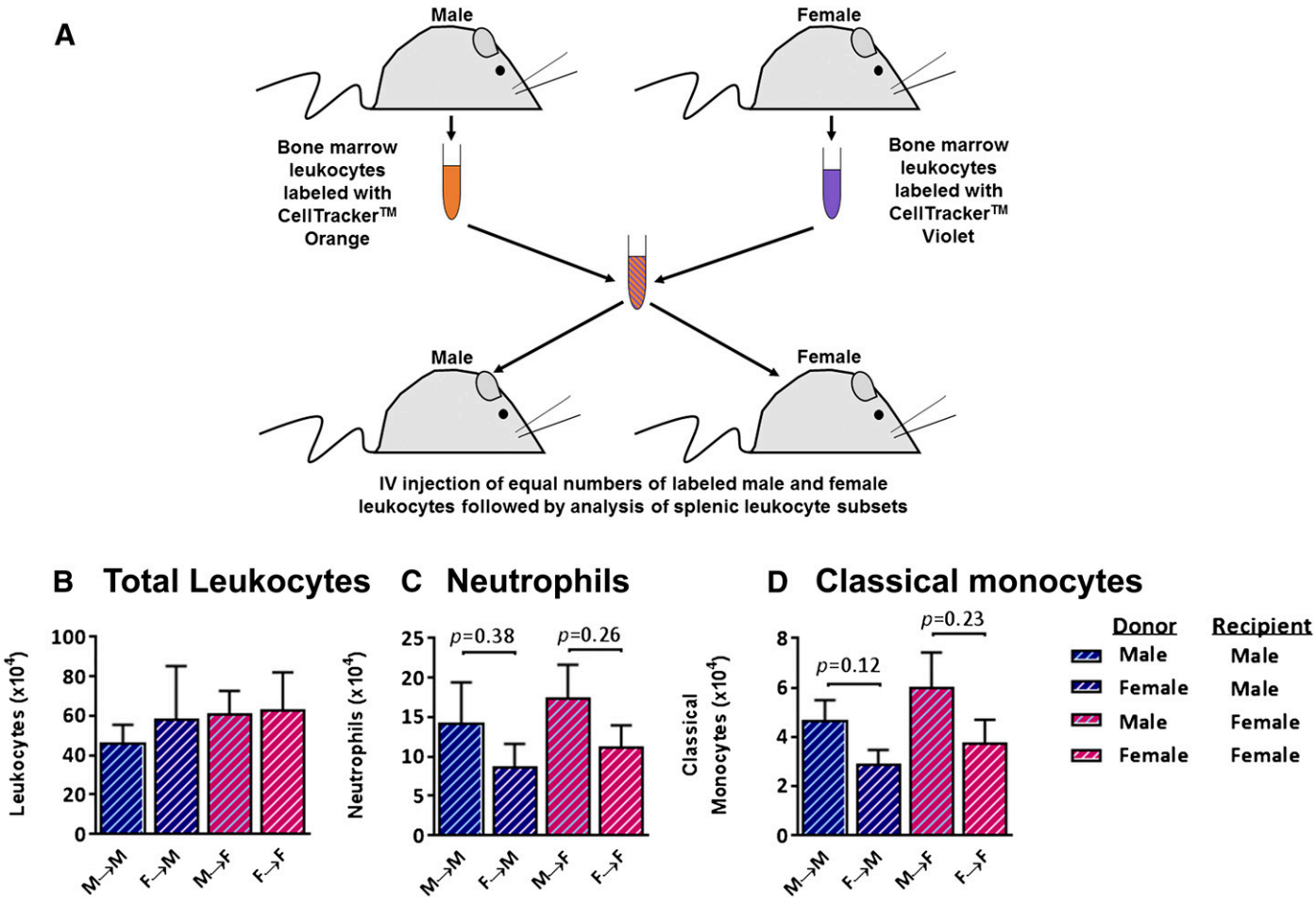


Figure 6. Trafficking of donor Tx leukocytes to recipient spleens. (A) Leukocytes were isolated from the BM of donor male and female C57BL/6 mice, and cells were labeled ex vivo with CellTracker Orange and CellTracker Violet dyes (2 μM; Life Technologies), respectively. Labeled donor leukocytes (10×10^6; 5×10^6 male + 5×10^6 female) were transferred (intravenously) into recipient male or female mice. After 4 h, recipient mice were culled by cervical dislocation and spleen leukocytes harvested. Leukocytes were counted on a hemocytometer and labeled with fluorescently conjugated antibodies to CD115 and Gr1. Samples were analyzed by flow cytometry. (B) Donor leukocytes in the recipient spleen. Male donor cells were identified as CellTracker Orange$^+$ and female donor cells as CellTracker Violet$^+$. (C) Donor neutrophils in the spleen were identified by subsequent gating on Gr1^{high} cells. (D) Donor classical monocytes in the spleen were identified by subsequent gating on CD115$^+$Gr1$^+$ cells. Data are means ± SEM, $n = 8/7/7/6$. Differences in the accumulation of donor leukocyte subsets in recipient spleens were determined by 1-way ANOVA with Bonferroni's posttest for multiple comparisons.

suggesting our observed sex differences in classical monocytes are not a result of the dimorphic neutrophil phenotype.

We hypothesized that like previous studies, the dimorphisms seen were a result of differences in immune cell phenotype and number. Whereas female mice did exhibit more peritoneal macrophages, we found no sex difference in the zymosan-sensing apparatus (TLR2, TLR6, dectin-1), contradictory to other studies, showing female macrophages to be higher in TLR2 [3]. Cytokines and chemokines also play a pivotal role in the immune response via the governing of leukocyte trafficking. As sex differences in cytokine production have been reported previously [29, 30], we examined the peritoneal microenvironment for differences in the ability of male and female mice to generate inflammatory mediators. Peritoneal cytokine microenvironments after zymosan were found to be comparable in male and female mice, suggesting equivalent chemotactic signal generation and providing no explanation for sex differences in recruitment. We show that the root of these sex differences in zymosan peritonitis is, in fact, upstream of the transmigration process, as males also had more circulating neutrophils and classical monocytes. Of note, the presence of these sex differences in the blood and tissue is also suggestive of similar transendothelial migration mechanisms and kinetics between the sexes. Collectively, a difference in the phenotype and function of resident peritoneal cells did not underlie the sex differences observed.

In response to inflammatory stimuli, leukocytes are mobilized from cellular storage pools, e.g., the BM, to participate in the inflammatory response [15, 31]. In line with previous studies [14], the BM indeed mobilized neutrophils in response to zymosan. We added to this mechanism

by demonstrating the same is true for monocytes. This storage pool was the major leukocyte contributor but released neutrophils and monocytes to equal extents in male and female mice. The spleen also represents a large site of leukocyte storage [32]. Importantly, we identify this organ as a dimorphic pool of leukocytes with respect to storage and trafficking. Male mice were found to have greater basal stores of neutrophils and monocytes in their spleen compared with female counterparts. Interestingly, spleen sizes were comparable in males and female mice, indicating that males do not have more splenic stores as a result of differences in organ size.

In response to zymosan, neutrophil and classical monocyte trafficking, with respect to the spleen, is inherently different, with mobilization of splenic classical monocytes in male mice and accumulation of neutrophils in the female spleen, and this potentially suggests that neutrophil and monocyte biology is distinct in the spleen (**Fig. 7**). In line with the observed classical

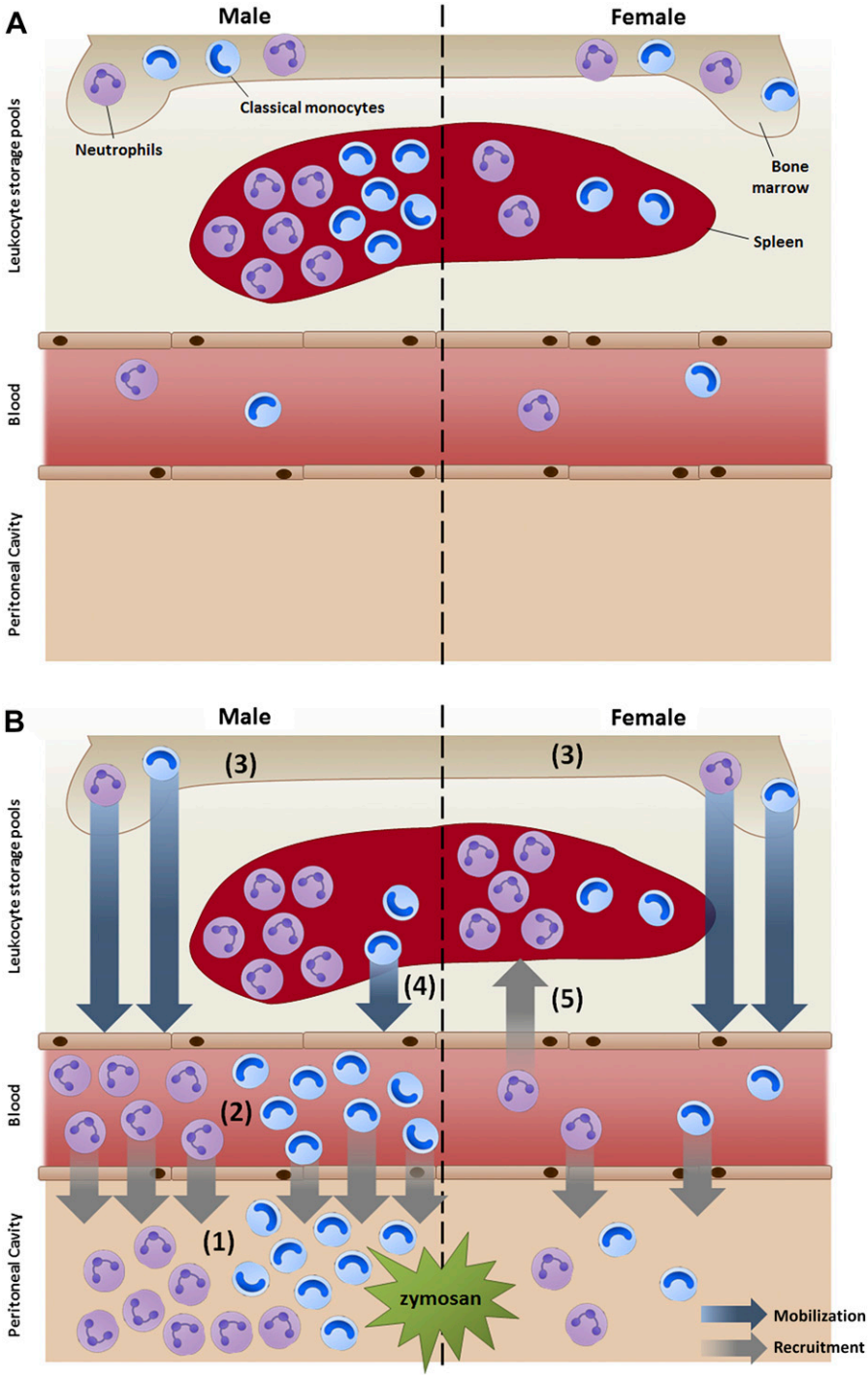


Figure 7. Sex differences in leukocyte trafficking in C57BL/6 mice in response to zymosan-induced peritonitis. (A) Under naïve conditions, there are equal numbers of leukocyte subsets between male and female C57BL/6 mice in the BM and the circulation, but not in the spleen, where males have higher numbers of neutrophils and classical monocytes than females. (B) During zymosan-induced peritonitis, more neutrophils and classical monocytes are recruited to the peritoneal cavity in males (1). In addition, circulating neutrophils and classical monocytes are more abundant in males (2); however, mobilization of these cells from the BM is the same between males and females (3). Interestingly, more classical monocyte mobilization from the spleen occurs in males (4), whereas greater numbers of neutrophils are recruited to the spleen in females (5).

monocyte mobilization in males, the spleen has been identified previously as a source of monocytes in a variety of inflammatory conditions, including atherosclerosis [17], myocardial infarction [16], and spinal cord injury [19], although the results in these studies did not discriminate by sex. The function of neutrophil trafficking in females is, however, unclear. Involvement of cross-talk with the adaptive immune system may provide an explanation. Whether these cells, in any way, contribute to the previously described splenic B helper neutrophils, which enhance innate Ig responses to microbial products would need to be investigated [33]. We hypothesize the traffic of neutrophils and classical monocytes to and from the spleen during zymosan peritonitis accounts for the overall sex differences observed in leukocyte recruitment, with splenic classical monocytes contributing to the additional recruited cells in male mice. This is supported by the fact that in splenectomized male mice, fewer classical monocytes and neutrophils are mobilized into the blood in response to zymosan peritonitis. Therefore, whereas the spleen has been implicated previously as a source of classical monocytes in certain inflammatory conditions, this is the first description of differential deployment of these cells in males and females.

In healthy individuals, monocytes and neutrophils are produced in the BM, yet the mechanisms resulting in the presence of mature classical monocytes in the spleen are unclear. We hypothesized that a more chemotactic spleen microenviroment or higher chemokine receptor expression may underlie the greater numbers of neutrophils and monocytes in the male spleen. This was not the case, as no major sex differences in the splenic cytokine/chemokine environment under homeostatic conditions were found. The expression of major neutrophil- and monocyte-associated chemokine receptors (CCR1, CCR2, CCR3, CCR5, CXCR2, CXCR4, C5a) was expressed at equivalent levels on male vs. female splenic neutrophils and classical monocytes. Against this trend was male nonclassical monocytes, which had greater expression of CCR2 but lower expression of C5aR1 and CXCR4. Higher CCR2 on male cells could provide explanation for more of these cells in the male vs. female spleen but leaves questions for the origins of the classical subset, which showed no differences in this receptor. The exact reasons for sex differences in nonclassical monocytes are unclear and may relate to the development of these cells. It is highly debated whether nonclassical monocytes are an independent monocyte subpopulation or whether they represent a classical monocyte precursor. Furthermore, whereas it is clear that a population of undifferentiated, bona fide monocytes resides in the spleen, it is not clear whether other populations exist to replenish the various splenic macrophage populations or whether these cells are derived from embryonic precursors and self renew [16, 34, 35].

Therefore, the mechanisms underlying the observed sex differences in splenic neutrophil and monocyte numbers remain unclear. Consequently, we reasoned that fundamental localization of such cells in specific organs under basal conditions is unlikely to be explained by a single molecule or receptor. Leukocyte Tx experiments revealed that there was not a significant difference in neutrophils and classical monocytes from male or female donors homing to the spleen of recipient mice. This was irrespective of the sex of the recipient and receiving spleen. These data suggest that there are indeed contributing factors from the "body", e.g., hormones, that are also influencing phenotype, function, and trafficking of neutrophils and classical monocytes. 17β-Estradiol is 1 such candidate, which has been reported to have "immune-damping" effects, particularly with respect to PRRs, adhesion molecules, and cytokine production [29, 36–38]. Furthermore, it may also be that these factors contribute to different rates of leukocyte hematopoiesis. Interestingly, nonclassical monocytes were detected only in negligible numbers in the spleens of recipient mice. This could be a result of different chemokine expression profiles of these monocytes, possibly as a result of a different maturity state compared with the classical subset, meaning that they are less amenable to splenic homing. Overall, the differences in the naïve mouse spleen are likely primarily a result of inherent differences between the male and female bodies.

Overall, this study has shown that sex differences in the inflammatory response are not restricted to neutrophil recruitment, extending to classical monocytes also. We have shown that the spleen represents a sexually dimorphic pool of leukocytes in C57BL/6 mice, and differences in splenic neutrophil and monocyte trafficking likely underlie differences in leukocyte recruitment in zymosan peritonitis. As C57BL/6 mice are used widely in characterizing inflammatory responses, the observations reported here are of paramount importance for the design and interpretation of experiments. Whether this is the case for other animal models and indeed for humans still needs to be determined. Should this work translate to the human, then the spleen leukocyte pool may well represent a therapeutic target for diseases, with aberrant leukocyte recruitment as the underlying pathogenesis.

AUTHORSHIP

E.K., R.S.S., and J.R.W. conceived of the work and designed the experiments. E.K., L.G.-G., and A.W. performed the experimental work and in conjunction with J.R.W., drafted the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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KEY WORDS:
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